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Osteopontin-dependent regulation of Th1 and Th17 cytokine responses in *Trypanosoma cruzi*-infected C57BL/6 mice

Miguel H. Santamaría [a], Ricardo S. Corral [b,*]

[a] *Laboratorio de Biología Experimental, Centro de Estudios Metabólicos, CP 39005 Santander, Spain*
[b] *Servicio de Parasitología-Chagas, Hospital de Niños "Ricardo Gutiérrez", Gallo 1330, C1425 Buenos Aires, Argentina*

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ABSTRACT

Osteopontin (OPN) is a multifunctional protein participating in the regulation of different Th cell lineages and critically involved in the initiation of immune responses to diverse pathogens. Our study goal was to verify whether OPN helps modulate the protective Th1 and Th17 cytokine responses in C57BL/6 mice infected with *Trypanosoma cruzi*, the etiological agent of Chagas disease. Parasite infection induced OPN release from murine macrophages in vitro and acute Chagas mice displayed enhanced serum levels of this cytokine at the peak of parasitemia. Upon administration of a neutralizing anti-OPN antibody, recently infected mice presented lower Th1 and Th17 responses, increased parasitemia and succumbed earlier and at higher rates to infection than non-immune IgG-receiving controls. The anti-OPN therapy also resulted in reduced circulating levels of IL-12 p70, IFN-γ, IL-17A and specific IgG_{2a} antibodies. Furthermore, antibody-mediated blockade of OPN activity abrogated the ex vivo production of IL-12 p70, IFN-γ and IL-17A, while promoting IL-10 secretion, by spleen macrophages and $CD4^+$ T cells from *T. cruzi*-infected mice. Th1 and Th17 cytokine release induced by OPN preferentially involved the $\alpha_v\beta_3$ integrin OPN receptor, whereas concomitant down-modulation of IL-10 production would mostly depend on OPN interaction with CD44. Our findings suggest that, in resistant C57BL/6 mice, elicitation of protective Th1 and Th17 cytokine responses to *T. cruzi* infection is likely to be regulated by endogenous OPN.

1. Introduction

Chagas disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, is one of the most important public health problems in Latin America. The overall prevalence of this human infection is estimated at 10 million cases, with 100 million at risk for infection [1]. The acute phase of infection is characterized by the presence of parasites in the bloodstream and diverse host tissues. Findings from several studies in experimental models of *T. cruzi* infection have suggested that a strong T helper type-1 (Th1) immune response is essential for the early control of parasitism [2]. Parasite replication is hampered by both innate and acquired immune responses mediated by macrophages, NK cells, B cells, $CD4^+$ and $CD8^+$ T cells [3]. Proinflammatory cytokines [e.g. interferon(IFN)-γ, tumor necrosis factor(TNF)-α and interleukin(IL)-12] also play a crucial role in protective immunity against *T. cruzi* [4]. IL-12 enhances IFN-γ production from NK cells and T cells. TNF-α cooperates with IFN-γ for induction of resistance to *T. cruzi* infection by activating phagocytes to release high levels of reactive nitrogen intermediates, such as NO, that are toxic to the parasite [3,5]. In addition, the proinflammatory cytokine IL-17A has recently been indicated as a necessary factor for the resolution of acute *T. cruzi* infection [6]. IL-17A is mainly produced by activated memory $CD4^+$ T cells, which are now classified as Th17 cells, and has been reported to elicit inflammatory responses through the organized production of inflammatory cytokines and chemokines, such as IL-1β, IL-6, TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-8/CXCL8, leading to the induction of leukocyte migration [7,8].

Osteopontin (OPN) is a multi-faced glyco-phosphoprotein synthesized by a variety of non-immune and immune cells and is implicated in interactions with cells mediating signaling, migration, and attachment [9,10]. Within the immune system, OPN is produced by activated T cells, NK cells, dendritic cells, and macrophages [11,12]. The existence of variant forms of OPN as a secreted (sOPN) and intracellular (iOPN) protein and its modification through post-translational events and proteolytic cleavage explain its broad range of functions. sOPN interacts with integrins and CD44, mediates cell adhesion and migration, and exhibits Th1 cytokine activities [13]. iOPN has been described to modulate the recruitment of innate immune cells, the secretion of IFN-α in plasmacytoid dendritic cells and the development of autoimmunity [14]. Both sOPN and iOPN participate in the regulation of different Th cell lineages [12]. Recent reports demonstrated that OPN deficiency may result in impaired clearing of intracellular infections,

* Corresponding author. Tel./fax: +54 1149634122.
E-mail address: ricardocorral56@hotmail.com (R.S. Corral).

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likely to be caused by a defective Th1 response. Analysis of mice lacking OPN revealed that this pleiotropic protein contributes to protection against a panel of intracytoplasmic pathogens, including herpes simplex virus type 1, *Listeria monocytogenes*, *Mycobacterium bovis* and *Plasmodium falciparum* [13,15–17]. OPN has been implicated in the development of pathogenic Th17-linked pathways [18] but data on Th17-cell protective immune responses that are mediated by OPN is not yet available. To our knowledge, no report has so far addressed the question about the immunoregulatory ability of OPN in chagasic infection. The aim of this study was therefore to verify the occurrence of OPN-dependent regulation of the protective Th1 and Th17 cytokine responses in *T. cruzi*-infected C57BL/6 mice.

2. Materials and methods

2.1. Mice

Female wild-type (WT) and OPN-deficient ($OPN^{-/-}$) C57BL/6 mice (6–8 weeks) were purchased from the Jackson Laboratory (Bar Harbor, USA) and housed under specific pathogen-free conditions. Each test group consisted of five ($OPN^{-/-}$) to eight (WT) mice. The protocol was approved by the local ethics committee for animal studies and followed the guidelines on accommodation and care of animals used for scientific purposes formulated by the European Commission (EU Directive 2010/63/EU).

2.2. Parasites and experimental infection

Mice were infected intraperitoneally with 10^3 blood-trypomastigote forms of the Y strain of *T. cruzi* [19]. The levels of parasitemia were evaluated by counting parasites in 5 μl of blood from the tail vein, and mortality was recorded daily [20]. Individual serum specimens were collected before and at different times after *T. cruzi* infection. For in vitro experiments, trypomastigotes of the Y strain were grown and purified from a monkey fibroblast cell line (LLC-MK_2). The parasite lysate preparation was obtained from freeze–thawed trypomastigotes, centrifuged at 10,000 g for 30 min, and filtered through a 0.22-μm-pore-size membrane filter.

2.3. Antibody treatment

In vivo blockade of OPN was achieved by using a neutralizing IgG antibody (OPN Ab), as reported previously [21]. Mice were treated with goat anti-mouse OPN Ab (50 μg/mouse, R&D Systems, USA) or control goat IgG 2 h before infection and every other day thereafter for 7 days by tail-vein injection.

2.4. Spleen cell, T cell and macrophage cultures

Single-cell suspensions of splenocytes from both uninfected and *T. cruzi*-infected C57BL/6 mice were prepared. Spleens were dispersed by gently teasing the spleen tissue through a 100-μm-pore-size nylon cell strainer. Erythrocytes were lysed by 2-min incubation in lysis buffer (150 mM NH_4Cl, 1 M $KHCO_3$, 0.1 mM EDTA, pH 7.2). Splenic $CD4^+$ T cells and macrophages were isolated using paramagnetic beads (Dynabeads® FlowComp™ Mouse CD4 and CD14 kits, respectively, Dynal; Invitrogen Corp., USA) as suggested by the manufacturer. Briefly, positive selection of cells was accomplished by incubating total splenocytes with the antibody mix and beads, followed by magnetic separation of bead-bound cells. Cell survival was 95 ± 2% as judged by trypan blue exclusion test. Surface marker staining using FITC-conjugated antibody to mouse CD4 (BD PharMingen, USA) or PE-labeled antibody to mouse F4/80 (AbD SeroTec, UK) and flow analysis were used to ensure adequate enrichment of isolated cell subsets. The purity of enriched cell preparations was: $CD4^+$, 98.6 ± 2.1 (mean% ± SD); $F4/80^+$, 97.1 ± 3.2. Leukocytes were washed twice and adjusted to 2×10^6 cells/ml in complete medium RPMI containing 10% fetal bovine serum (FBS), 1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded by triplicate in 24-well plates (Sarstedt, Germany) in the presence of 10 μg/ml of parasite lysate or medium alone. All the media and reagents were endotoxin-free. For intracellular cytokine staining, splenic lymphocytes (5×10^5) were stimulated with parasite lysate (10 μg/ml) for 24 h. BD GolgiStop (BD Pharmingen) was added at the final 6 h. The cells were first stained extracellularly with FITC-conjugated anti-CD4 (RM4-5) and PE-Cy5-conjugated anti-CD3 (17A2), then fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Pharmingen). The cells were then separately stained intracellularly with PE-conjugated anti-IFN-γ (XMG1.2), anti-IL-17A (TC11-18H10) or isotype control antibodies (all from BD Pharmingen). Samples were acquired on a FACSCalibur (BD Biosciences) and data were analyzed using BD CellQuest Pro software (BD Biosciences). To test the effect of OPN on splenocytes, purified $CD4^+$ T cells were cultured in the presence or absence of 1 μg/ml of mouse recombinant OPN (rOPN, R&D Systems) and infected with *T. cruzi* culture-derived trypomastigote forms at a 5:1 parasite:host cell ratio. In some experiments, $CD4^+$ T cells were stimulated with rOPN plus recombinant mouse IL-12 (100 ng/ml, R&D Systems) before infection. To study the involvement of OPN receptors, $CD4^+$ T cells and macrophages were treated in parallel experiments with 5 μg/ml of receptor-blocking monoclonal antibodies, including anti-CD44 and anti-β3 integrin (BD Biosciences). Supernatants were collected after 72 h at 37 °C and stored at −70 °C.

Normal mouse inflammatory macrophages were harvested from the spleens and peritoneal cavities 3 days after the injection of 1 ml of 3% sodium thioglycolate (Sigma–Aldrich, USA). The adherent cells were obtained after a 2- to 4-h incubation of single-cell suspensions in 96-well plates at 37 °C. The non-adherent cells were removed by exhaustive washing with Hank's medium. Moreover, primary cultures of bone marrow-derived macrophages (BMM) were obtained from femurs of naïve C57BL/6 mice as described [22] and cultured in BMM medium (DMEM supplemented with 20% FBS and 10% 3T3 fibroblast supernatant containing macrophage-CSF. The cells were differentiated for 7 days yielding > 98% $Mac3^+$, $F4/80^+$ and $CD11b^+$ BMM (data not shown). Macrophages were polarized with either 100 ng/ml LPS + 100 U/ml IFN-γ (M1) or 5 ng/ml IL-4 (M2). Non-polarized BMM (M0) were cultured in complete medium alone. Culture trypomastigotes were added in a 5:1 parasite-to-cell ratio and incubated for 48 h at 37 °C. The supernatants were then harvested, filtered and stored at −70 °C.

2.5. Measurement of murine cytokines and T. cruzi-specific antibodies

OPN, IFN-γ, IL-12 p70, IL-10, IL-13 and IL-17A measurements were done by sandwich ELISA (R&D Systems) according to the manufacturer's specifications. Supplied standards were used to generate the standard curves.

Serum specific IgG antibody response in parasite-infected mice was measured by use of a commercial ELISA kit (bioMérieux, France), as reported elsewhere [23]. To identify the antibody isotypes, the reaction was revealed with peroxidase-conjugated rat monoclonal antibodies for mouse IgG_{2a} and IgG_1 subclasses (BD Pharmingen), which are routinely used as an indirect measure of Th1 and Th2 immune responses, respectively [24].

2.6. Statistical analyses

Data analysis was carried out using Prism 4.0 software (GraphPad Software, USA). The results are expressed as the mean ± SD. Studenťs *t*-test and one-way analysis of variance (ANOVA) with a *post hoc* Tukey test were used to determine the statistical significance of the intergroup comparisons. Mann–Whitney-U–Wilcoxon and chi-square logrank tests were conducted for the analyses of parasitemia and survival curves, respectively. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Live T. cruzi trypomastigotes induce OPN production by adherent splenocytes, peritoneal macrophages and BMM

Although a variety of cell types express OPN, macrophages are a major source [25,26]. As phagocytes are among the first cells to be parasitized by *T. cruzi*, we therefore investigated whether trypomastigotes could trigger OPN production by macrophages obtained from different mouse tissues. After 48 h of infection, adherent splenocytes secreted levels of OPN similar to those of whole spleen cells and significantly higher than those detected in uninfected cell supernatants (Table 1). This effect can be attributed to an increase of OPN production per cell, as infected cultures did not present a significant expansion of the OPN-secreting splenocyte subset (data not shown). Inflammatory peritoneal macrophages and BMM (M1 and M2 subpopulations) also showed enhanced OPN release when stimulated with live *T. cruzi* trypomastigotes. In contrast, nonadherent spleen cells produced low amounts of this cytokine upon parasite infection in vitro. Besides, trypomastigote invasion promoted IL-12 p70 production by adherent splenocytes and peritoneal inflammatory and bone marrow-derived (M0 and M1 subpopulations) macrophages (Table 1). These data provide evidence that *T. cruzi* is capable of readily inducing OPN biosynthesis, accompanied by enhanced IL-12 p70 secretion, in murine macrophages.

3.2. Endogenous OPN contributes to host resistance to T. cruzi infection in vivo

Since we have found that OPN is produced by macrophages after stimulation with *T. cruzi* trypomastigotes in vitro, we next evaluated whether this cytokine had any effect on the course of acute murine infection. Inbred C57BL/6 mice infected with the *T. cruzi* Y strain are known to behave as relatively resistant with respect to the magnitude of parasitemia and mortality rate [27]. In our series, mice were each inoculated with OPN neutralizing Ab 2 h before and on days 3, 5 and 7 of infection. A group of control mice were also infected and received purified goat IgG at the same time points. Parasitemia was determined in both OPN Ab- and non-immune IgG-treated animals. The anti-OPN therapy resulted in a significantly higher parasitemia on days 10 and 11 post-infection

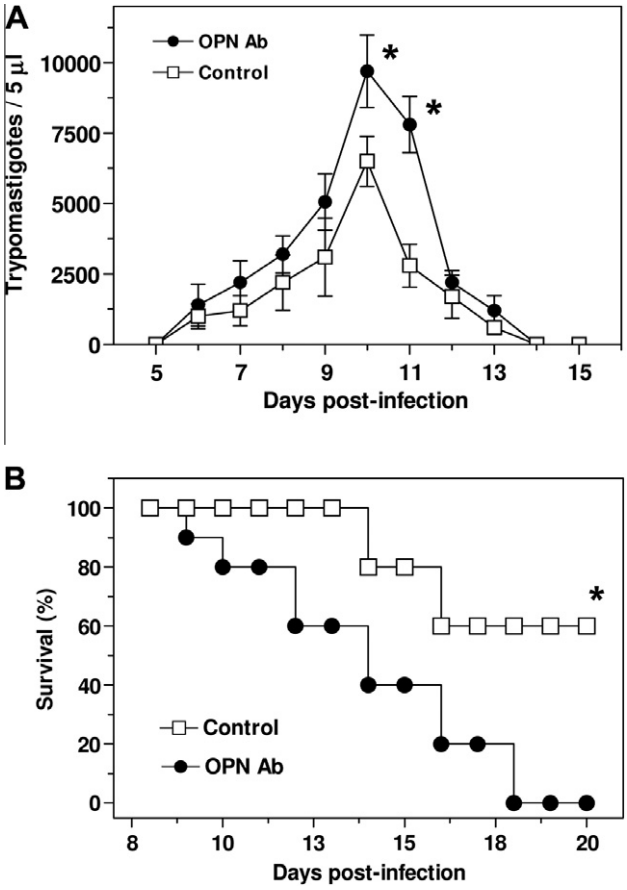

Fig. 1. Natural course of murine *T. cruzi* infection, as measured by parasitemia (A), and survival rate (B). C57BL/6 mice were infected with 10^3 blood-trypomastigote forms of the Y strain and treated with OPN neutralizing antibody (OPN Ab, *filled circle*) or non-immune goat IgG (Control, *open square*) 2 h before infection and again on days 3, 5 and 7 after infection. In panel (A), the mean ± SD of parasites per 5 µl of fresh blood for three independent experiments (8 mice/group) is shown. In panel (B), the percent survival for each group was determined daily. $^{*}P < 0.05$.

Table 1
T. cruzi trypomastigotes induce OPN and IL-12 p70 production by adherent splenocytes, inflammatory peritoneal macrophages and bone marrow-derived macrophages.

Cell type[a]	OPN (ng/ml)		IL-12 p70 (pg/ml)	
	Uninfected	Infected	Uninfected	Infected
Spleen cells				
Total	311 + 44	1514 + 301*	246 + 37	1911 + 82**
Adherent	277 ± 78	997 + 191*	193 + 92	1820 + 155**
Nonadherent	15 ± 12	19 ± 24	20 + 13	41 ± 20
Peritoneal exudate cells				
Inflammatory macrophages	206 ± 52	1144 + 217*	289 + 75	2808 + 132**
Bone marrow-derived cells				
Nonpolarized macrophages (MO)	53 ± 42	345 ± 169	49 + 28	1214 + 294**
M1 polarized macrophages	405 ± 88	1299 + 333*	436 + 94	3372 + 119**
M2 polarized macrophages	312 + 91	986 + 225*	108 + 63	155 + 47

[a] The different cell populations were obtained and incubated with live trypomastigote forms at a 5:1 parasite-to-cell ratio for 48 h, and the OPN and IL-12 p70 production was then assayed by ELISA in cell supernatants. Uninfected cells were included as controls. The results are expressed as means ± SD of triplicate analyses.
* $P < 0.05$ between infected and uninfected cultures..
** $P < 0.01$ between infected and uninfected cultures.

(Fig. 1A). More importantly, Ab-mediated blockade of OPN activity led to increased and earlier mortality in infected C57BL/6 mice (Fig. 1B). These experiments establish the relevance of endogenous OPN in controlling acute *T. cruzi* murine infection.

3.3. OPN is involved in Th1- and Th17-mediated immunity against T. cruzi infection

The ability of OPN to regulate Th1/Th17 immunity in C57BL/6 mice acutely infected with *T. cruzi* was determined ex vivo by intracellular cytokine analysis of splenic $CD4^+$ T cells using a parasite lysate preparation for restimulation. As shown in Fig. 2A, there was an expansion of both Th1 ($CD4^+$ IL-$17A^-$IFN-γ^+) and Th17 ($CD4^+$ IL-$17A^+$IFN-γ^-) cell subpopulations at 7 days of infection compared to splenocytes from the uninfected group. Remarkably, in vivo administration of OPN Ab to infected mice abrogated the increase in the proportion of Th1 and Th17 cells recorded during early Chagas disease. To directly demonstrate the effect of OPN on splenic lymphocytes, isolated $CD4^+$ T cells were infected in vitro with culture trypomastigotes, further stimulated with mouse rOPN and cytokine production was measured. Upon *T. cruzi* infection, rOPN strongly triggered both IFN-γ and IL-17A secretion into the cell supernatant (Fig. 2B). As expected, addition of exogenous IL-12 to OPN-stimulated *T. cruzi*-infected cultures boosted IFN-γ, but not IL-17A, production further (results not shown). To further confirm that OPN plays an essential role in modulating Th1 and Th17 cytokine responses during experimental *T. cruzi* infection, we analyzed the ex vivo release of IFN-γ and IL-17A upon antigen stimulation of splenic $CD4^+$ T cells from uninfected and parasite-infected (10 dpi) WT and OPN null C57BL/6 mice. In

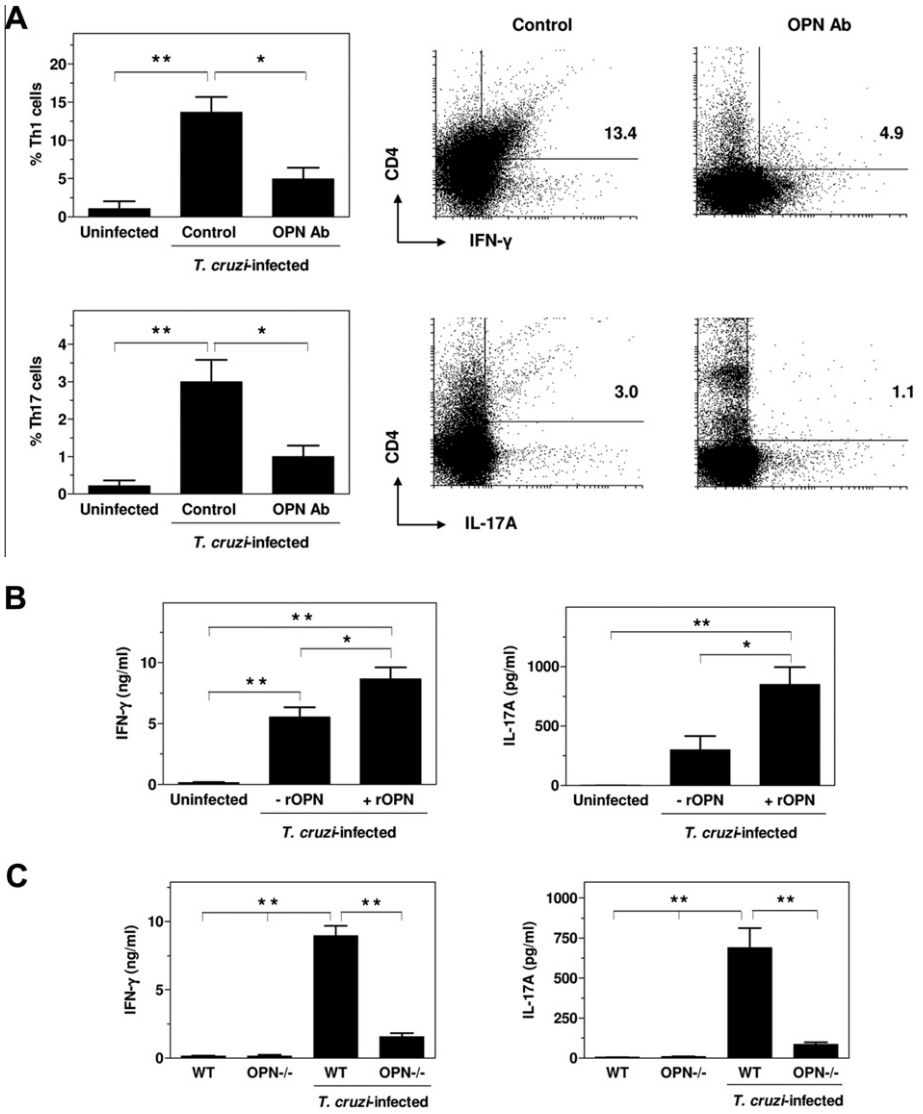

Fig. 2. Increased numbers of IFN-γ- and IL-17A-producing (Th1 and Th17, respectively) cells, and production of IFN-γ and IL-17A in C57BL/6 mice infected with *T. cruzi*. (A) Splenocytes were prepared from uninfected mice and infected mice (10 days post-infection) treated with OPN neutralizing antibody (OPN Ab) or non-immune goat IgG (Control). The cells were stained ex vivo for intracellular cytokines and the percentage of $CD4^+$ IFN-γ^+ (Th1) and $CD4^+$ IL-$17A^+$ (Th17) cells was determined by flow cytometry (representative plots are shown). The results (mean ± SD for the different groups; $n = 8$ mice per group) shown are representative of three independent experiments with similar outcome. $^*P < 0.05$; $^{**}P < 0.01$. (B) $CD4^+$ T cells were isolated from the spleen of naive mice, then cultured in the presence or absence of 1 μg/ml of mouse recombinant OPN (rOPN) and infected with *T. cruzi* trypomastigote forms at a 5:1 parasite:host cell ratio for 72 h. The levels of IFN-γ and IL-17A in the culture supernatant fluids were determined by ELISA. Data (mean ± SD of triplicate determinations) are representative of four independent experiments. $^*P < 0.05$; $^{**}P < 0.01$. (C) $CD4^+$ T cells were isolated from the spleen of *T. cruzi*-infected (10 dpi) wild-type (WT) or OPN knockout ($OPN^{-/-}$) C57BL/6 mice and cultured in the presence of parasite lysate (10 μg/ml) for 72 h. Splenic $CD4^+$ T cells from uninfected WT and $OPN^{-/-}$ mice were also assayed. The levels of IFN-γ and IL-17A in the culture supernatant fluids were determined by ELISA. The results (mean ± SD for the different groups; $n = 5$ mice per group) shown are representative of two independent experiments with similar outcome. $^{**}P < 0.01$.

supernatants of CD4$^+$ T cells from infected *OPN*$^{-/-}$ animals, the levels of both cytokines were far lower than those produced by cells from infected WT mice (Fig. 2C). Altogether, these findings suggest that OPN is capable of positively regulating both Th1 and Th17 cytokine responses induced in parasite-infected immune cells.

In the light of these results, we examined by ELISA the profile of proinflammatory cytokines and the isotype pattern of *T. cruzi*-specific antibodies in the sera of experimentally infected mice. First, we analyzed kinetics of serum OPN during the acute phase of parasite infection. At the peak of parasitemia (10 days post-infection, dpi), circulating levels of this cytokine were significantly elevated above basal value and gradually declined with time (Fig. 3A). Since OPN is known to stimulate macrophages to produce IL-12, which in turn may induce IFN-γ release by different immune cells, we next measured serum concentrations of these proinflammatory mediators in acutely infected mice. As shown in Fig. 3B and C, *T. cruzi* infection (10 dpi) increased the bloodstream levels of IFN-γ and IL-12 (p70 subunit). Furthermore, infected mice showed a Th1-biased (IgG_{2a} to IgG_1 ratio = 1.57) humoral response to *T. cruzi* (Fig. 3D). Interestingly, the serum IL-17A levels also augmented at the peak of the parasitemic phase of infection (Fig. 3E). On the other hand, infected mice receiving a blocking antibody to OPN presented significantly reduced concentrations of serum IL-17A, IL-12 p70 and IFN-γ, as well as a marked decrease of the Th1-associated *T. cruzi* antibody reactivity (IgG_{2a} to IgG_1 ratio = 0.81) (Fig. 3B–E).

We also measured the ex vivo production of IFN-γ, IL-12 p70, IL-10, IL-13 and IL-17A by T lymphocytes and macrophages purified from spleen of acutely (10 dpi) *T. cruzi*-infected and OPN Ab- or goat IgG-treated mice upon stimulation with trypomastigote antigens for 72 h (Fig. 4). CD4$^+$ T cells and macrophages isolated from uninfected mice secreted very low amounts of IFN-γ and IL-17A, and IL-12 p70, respectively. Parasite infection mostly induced a mixed Th1/Th17 cytokine response in C57BL/6 mice. ELISA results revealed that, compared with the control IgG group, CD4$^+$ T cells from the OPN Ab group produced significantly lower levels (11,667 ± 3512 vs 5102 ± 1990 pg/ml, respectively) of IFN-γ, the emblematic cytokine for inflammatory and cell-mediated responses, and the canonical Th17 cytokine IL-17A (304 ± 121 vs 148 ± 36 pg/ml) (Fig. 4A and C). Accordingly, macrophage supernatants from OPN Ab-receiving mice presented significantly less concentration (353 ± 41 pg/ml) of IL-12 p70, a potent inducer of IFN-γ production by several cell types, than that detected in the non-immune IgG-treated animals (969 ± 96 pg/ml) (Fig. 4B). In contrast, parasite antigen-stimulation of CD4$^+$ T cells from the OPN Ab group rendered enhanced release (808 ± 19 pg/ml vs 512 ± 65 pg/ml from

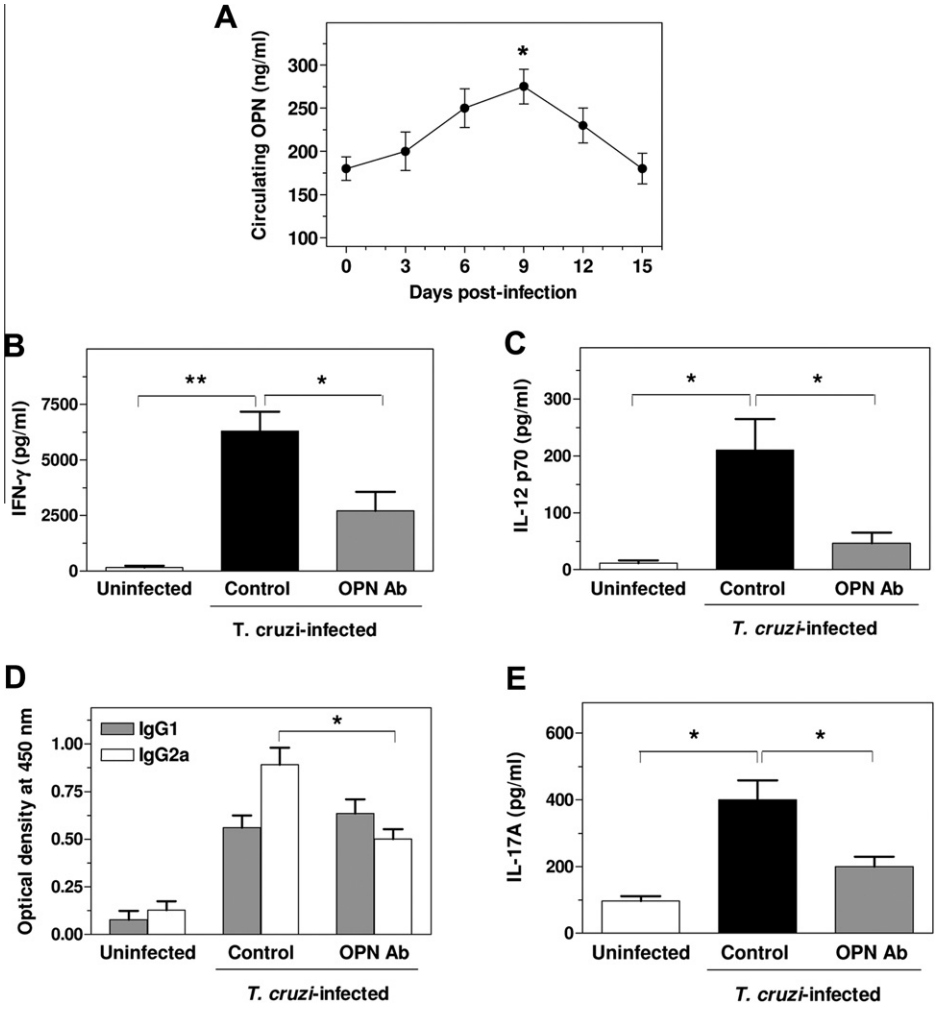

Fig. 3. Levels of proinflammatory cytokines and isotype pattern of *T. cruzi*-specific antibodies in the sera of mice infected with *T. cruzi* and treated with OPN neutralizing antibody (OPN Ab) or non-immune goat IgG (Control). (A) Kinetics of serum OPN response determined by ELISA in acutely infected mice. (B–E) Circulating cytokines and humoral immune response in chagasic mice. Mouse sera were collected on day 10 of infection and were assayed in triplicate by capture ELISA for IFN-γ (B), IL-12 p70 (C), *T. cruzi*-specific IgG antibodies (IgG_1 – *grey bars* – or IgG_{2a} – *white bars* – isotype) (D) and IL-17A (E) as described under materials and methods. Specimens collected from uninfected mice were included in the assay. Each bar represents the mean ($n = 8$) ±SD for the different groups. Similar results were obtained in two additional experiments. $^*P < 0.05$; $^{**}P < 0.01$.

normal goat IgG-inoculated mice) of IL-10, a counter-regulator of IFN-γ [28] (Fig. 4D). The levels of the Th2 cytokine IL-13 remained unchanged in *T. cruzi*-infected or OPN Ab-treated cultures (Fig. 4E). We next asked which functional receptor (CD44 or $\alpha_v\beta_3$ integrin) was responsible for the observed regulation of cytokine production by OPN. Induction of IFN-γ, IL-12 p70 and IL-17A was significantly inhibited by monoclonal antibody to the integrin β_3 subunit, but not by antibody to CD44 (Fig. 4A–C). On the other hand, OPN-dependent inhibition of IL-10 secretion was blocked by antibody to CD44 but not by antibody to integrin β_3 (Fig. 4D). Taken together, these experiments underline the importance of endogenous OPN in regulating the development of Th1 and Th17 cytokine responses against acute *T. cruzi* infection, mostly through interaction with its integrin receptor.

4. Discussion

The elicitation of regulated immune pathways is necessary for adequate protection against the growth of many infectious agents, including the protozoan parasite *T. cruzi* [2,29]. Consequently, elucidation of the underlying mechanisms will help us to understand how normal protective responses differ from pathogenic ones that result in exacerbated inflammation and self-reactivity. OPN is currently emerging as a key regulator of immune cell biology [30]. Among its multiple actions, OPN is capable of polarizing the response towards Th1-biased status as demonstrated previously by the induction of IFN-γ release by activated Th1 cells and the up-regulation of IL-12 production while inhibiting IL-10 expression in macrophages [13,31,32]. Additionally, OPN promotes IL-17A

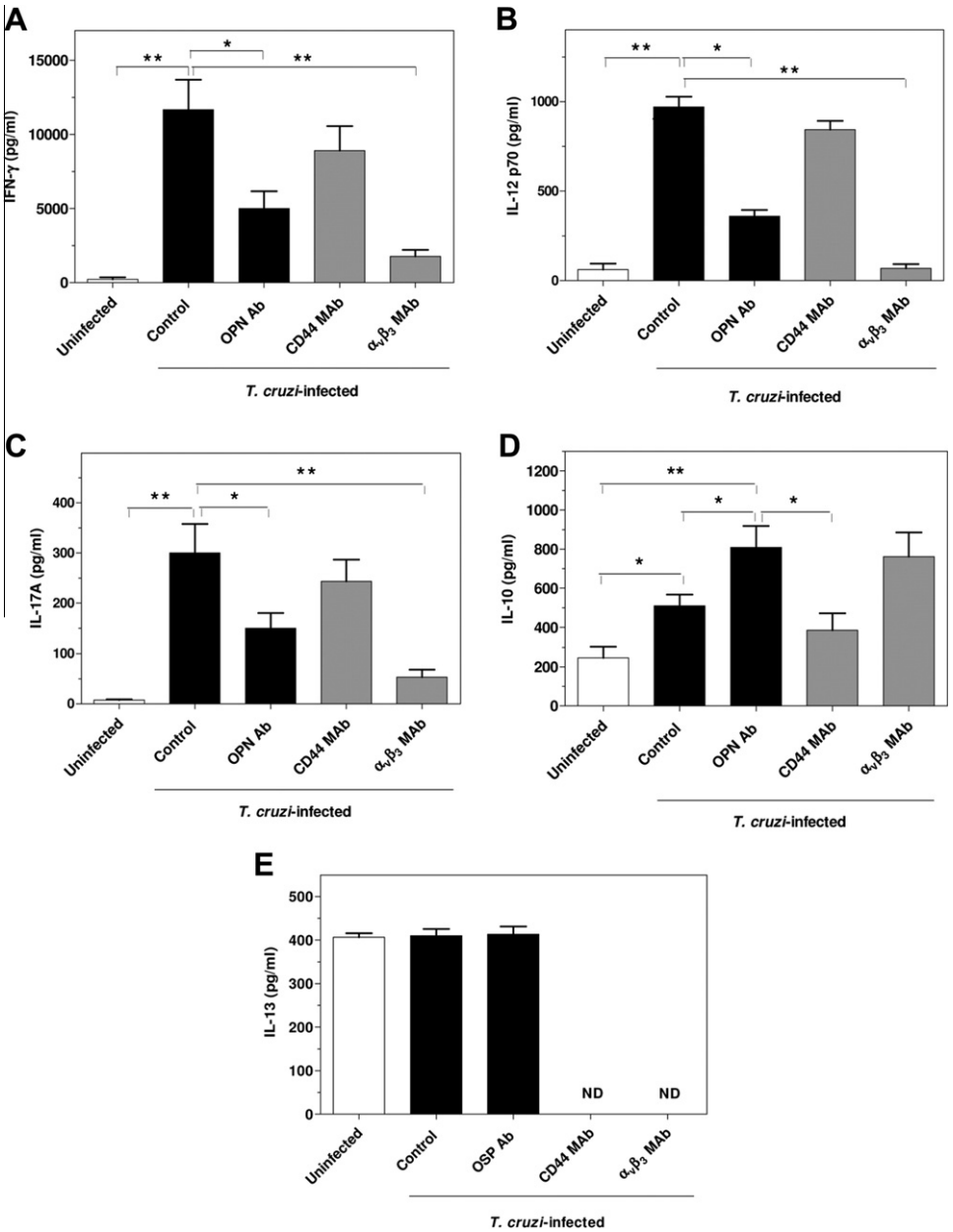

Fig. 4. Ex vivo analysis of cytokine production profile in response to trypomastigote antigens in *T. cruzi*-infected mice treated in vivo (*black bars*) with OPN neutralizing antibody (OPN Ab) or non-immune goat IgG (Control). Splenocytes were isolated from C57BL/6 mice at 10 days post-infection. Uninfected mice (*white bars*) were also analyzed. Purified $CD4^+$ T cells and macrophages were stimulated with 10 μg/ml of parasite lysate for 72 h. In parallel experiments, the cells were OPN-stimulated and treated ex vivo (*grey bars*) with 5 μg/ml of anti-CD44 or anti-β3 integrin receptor-blocking monoclonal antibodies. Culture supernatants were assessed for the concentration of the indicated cytokines: (A) IFN-γ, (B) IL-12 p70, (C) IL-17A, (D) IL-10, and (E) IL-13, using ELISA assays. Values represent mean concentration (pg/ml ± SD) of triplicates. Results were reproduced in three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$. ND, not determined.

expression in Th17 lymphocytes [33]. In OPN-deficient mice, impaired clearance of intracytoplasmic microorganisms has been associated with abrogation of IL-12 secretion and enhanced synthesis of IL-10 [13]. Nevertheless, the protective ability of OPN against infection does not extend to all types of pathogen [34]. In view of the central role played by the inflammatory cytokine activity in the early resistance against chagasic infection, in this work we examined whether OPN participates in mounting efficient Th1 and Th17 anti-*T. cruzi* responses.

The innate immune system seems to have a fundamental position in at least two important aspects of Chagas disease: the control of parasite replication and spread in host tissues, and the inflammatory reaction in infected target organs. Phagocytic cells begin to release TNF-α and IL-12 soon after *T. cruzi* infection in vivo, thus contributing to protect the host against a lethal infection [35]. We herein demonstrated that live trypomatigotes are able to induce OPN secretion by normal spleen, peritoneal and bone marrow-derived macrophages, and also detected significantly enhanced OPN levels in the sera from infected mice at the time of maximum parasite load. Akin to what has been shown for other intracellular microbes [36,37], these findings indicate that *T. cruzi* triggers transient OPN production in the host during the parasitemic phase of infection.

In an attempt to understand the effects of parasite-induced OPN on resistance to *T. cruzi*, a function-blocking OPN Ab was administered to C57BL/6 mice immediately before and repeatedly after infection. Our results suggest that OPN has the ability to positively regulate the proportion of Th1 and Th17 cells generated and the amount of cytokine produced by each subset upon infection. Remarkably, during the course of experimental Chagas disease, we observed that the mice treated with the neutralizing antibody became more susceptible to *T. cruzi* as evidenced by increased parasite loads and higher and earlier death rates, in comparison with those of control IgG mice. Since OPN depletion may lead to defective host defence against intracellular pathogens [16,17], we further analyzed the serum antibody and cytokine repertoire exhibited by the experimental groups at the peak of parasitemia. Acute chagasic infection induced a mixed Th1/Th17 cytokine profile in C57BL/6 mice. Administration of the OPN blocking Ab to infected animals resulted in a significant decline in the levels of IL-12 p70, IFN-γ, IL-17A and anti-*T. cruzi* IgG_{2a}, suggesting that in vivo elicitation of protective Th1/Th17 immunity to the parasite is likely to be dependent on endogenous OPN.

Upon antigen stimulation, $CD4^+$ T cells from infected *OPN* null mice released low amounts of both IFN-γ and IL-17A, suggesting OPN involvement in Th1 and Th17 cytokine production. Moreover, splenic $CD4^+$ T cells and macrophages from OPN Ab-treated mice stimulated in vitro with parasite lysate presented reduced secretion of IL-12 p70, IFN-γ and IL-17A, with a concomitant increase in IL-10 production, as compared with non-immune IgG group. Previous studies have determined that OPN dampens macrophage expression of IL-10 and induces the production of IL-12, further stimulated by IFN-γ derived from activated T cells [38]. Also, OPN has been shown to amplify IL-17 release and Th17 cell differentiation [33,39]. Accordingly, our results point out that in experimental acute Chagas disease endogenous OPN is implicated in the up-regulation of the levels of IL-12 p70, IFN-γ and IL-17A, all relevant agents for host defence against *T. cruzi* [40], and also in the down-regulation of IL-10, an interleukin previously shown to inhibit Th1/Th17 cytokine synthesis [28,41]. Therefore, OPN sets the stage for an efficient control of parasite infection through differential pro-and anti-inflammatory cytokine responses. In this regard, a bulk of studies substantiates that Th1-associated cytokines are essential elements in early resistance against acute Chagas disease [2], whereas Miyazaki et al. [6] and Mou et al. [42] revealed that IL-17 is important for the successful resolution of recent *T. cruzi* and *T. congolense* infection in mice, respectively. However, balancing anti-*T. cruzi* immunity is critically required as disproportionate IFN-γ and IL-17 production can promote pathogenesis as demonstrated [43,44].

In agreement with previous findings in murine intracellular infections and autoimmune encephalomyelitis [13,33], our results suggest that the secretion of IL-12 p70, IFN-γ and IL-17A induced by OPN during acute chagasic infection preferentially involved the $\alpha_v\beta_3$ integrin OPN receptor. On the other hand, CD44 engagement seems to be implicated in OPN-dependent inhibition of IL-10 production by $CD4^+$ T cells. However, differences in the OPN receptor usage that may be traced to the distinct experimental settings and use of animal models, have been documented [39] and further studies are needed to unravel the precise mechanisms by which OPN exerts its immunomodulating effect.

In conclusion, as reported for other intracellular pathogens [13,16,17], our data suggest that endogenous OPN represents an important regulatory factor of the protective Th1 and Th17 responses in *T. cruzi*-infected C57BL/6 mice. These findings fill a gap in our comprehension of the early molecular events that allow full maturation of Th1/Th17 immunity to *T. cruzi*.

Competing interests

None.

Ethics approval

This study was conducted with the approval of the local ethics committee.

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